Appearance of Endoproteolytic Enzymes during the Germination of Barley

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ABSTRACT

Barley endoproteolytic enzymes are important to germination because they hydrolyze endosperm storage proteins to provide precursors for new protein synthesis. We recently developed an electrophoretic method utilizing gel Incorporated protein substrates to study the endoproteases of 4-d-germinated barley (Hordeum vulgare L. cv Morex) grain. This work extends those findings to determine the temporal pattern of the appearance of the endoproteases during germination, the sensitivities of the proteases to class-specific proteinase inhibitors, and where, in germinating aleurones, the proteinases reside. Six endoprotease activity bands (representing a minimum of seven enzymes) were present in 5-d-germinated barley grain extracts subjected to electrophoresis in nondenaturing gels at pH 8.8. The activities of two of the enzyme bands ("neutral" proteinases) increased as the pH was increased from 3.8 to 6.5. The activities of the remaining four ("acidic") bands diminished abruptly as the pH increased above 4.7. Two proteinase bands hydrolyzed gelatin but not edestin, four of the proteinases hydrolyzed both gelatin and edestin at nearly the same rates, and one enzyme degraded only edestin. One neutral endoprotease was sensitive to diisopropyl fluorophosphate inhibition, and the other was not inhibited by any of inhibitors tested. Four of acidic enzymes were cysteine proteinases (inhibited by trans-epoxysuccinyl-l-leucylamidod(4-guanidino)butane and N-ethylmaleimide); the other was an aspartic acid endoprotease (sensitive to pepstatin). Only the aspartic proteinase was detected in either ungerminated or steeped barley grain. During the germination (malting) process, the aspartic endoprotease activity decreased until the second day of germination and then increased until germination day 5. The first endoprotease(s) induced during germination was a neutral enzyme that showed activity on the 1st day of the germination phase after steeping. Most of the endoproteases became active on the 2nd or 3rd germination day, but one cysteine proteinase was not detected until the 5th day. Acid cysteine proteinases were present in the aleurone, scutellum, and endosperm tissues but not in shoots and roots. The aleurone layer and endosperm contained almost exclusively band B1 neutral proteinases, whereas the scutellum, shoots, and roots contained both B1 and B2 bands. This work shows that germinating barley contains a complex set of proteinases whose expression is temporally and spatially controlled. But, at the same time, it also shows that this electrophoretic method for separating and studying individual enzymes of this complex will allow us to more readily characterize and purify them.

During barley germination, the developing embryo obtains its nutrition mainly from the starchy endosperm, which has been described (19) as corresponding to a "huge secondary lysosome: it contains a large battery of acid hydrolases ... and its only function is complete hydrolysis of its contents to provide building block molecules for use in other tissues." Among the hydrolases are the endoproteases, which are responsible for catalyzing the first steps in the digestion of barley (Hordeum vulgare) storage proteins. They provide soluble polypeptide substrates that the exoproteases reduce to free amino acids, which are readily utilized by the embryo.

Several parallel processes take place during barley germination: there is a decrease in the content of insoluble storage proteins (15, 22, 23), an increase in the peptide transport activity of the scutellum (28), a possible decrease in the endogenous proteinase inhibitor activity (18), and an increase in the endoproteolytic activity of grain extracts (1, 19).

An aspartic acid endoprotease has been found in ungerminated barley (20, 27). This enzyme has been purified and partially characterized and appears to be related to animal cathepsin D (25).

There is a strong increase in the total proteolytic activity of germinating barley that begins at about the 3rd day of germination (1, 19). The enzymes responsible for this increased activity were reportedly cysteine proteinases active at acidic pH values (5, 18). Two of these enzymes have been purified. One, with Mr 30,000, was isolated from 4-d-germinated barley grain (22). The second, Mr 37,000, was isolated from the medium upon which embryoless barley half seeds, stimulated by GA3, were incubated (12).

However, it is well known that there are more than these three endoproteases in germinating barley. Enari and Mikola (5) found that there were at least two cysteine proteinases and three metal-requiring enzymes. A study by Burger et al. (4) also revealed five enzymes, two "neutral" proteinases and three other "acidic" enzymes.

There are still many questions about barley endoproteases that have not yet been answered, such as (a) how many enzymes are present in germinating and germinated barley, (b) what are their substrate preferences and sensitivities to class-specific inhibitors, and (c) which are involved in hydrolyzing hordeins, the main barley storage proteins?

We recently concluded a study using electrophoresis with non-denaturing gels containing incorporated substrate proteins that revealed that there were seven proteolytic activity bands in a crude green malt (4-d-germinated barley) extract.
Five of the endoproteinase bands were maximally active at pH 3.8, and the other two were maximally active at pH 5.5 to 6.5. On the basis of these findings, we chose the pH values 3.8 and 6.5 for these inhibitor studies presented in this paper. The endoproteinases most active at pH 3.8 are termed acidic enzymes because they are most active at acidic pH values. Enzymes active at pH 6.5 are designated neutral proteinases. Most of acidic enzymes hydrolyzed both gelatin and edestin, neutral enzymes degraded only gelatin, and the proteinase present in steeped barley could only be detected using edestin (29). For this reason, both of these substrates were utilized. Hordein was also tested as a substrate because its enzymic hydrolysis is of major importance to the germination process. The work described in this paper was conducted to further characterize these enzymes, to determine the temporal pattern of the appearance of the endoproteinases during germination, and to ascertain what proteinase classes they belong to and where the various proteinases reside in the germinating grain.

MATERIALS AND METHODS

Materials

Gelatin, edestin, hemoglobin, cysteine, DTT, EDTA, Triton X-100, Tris, glycine, DIC², DFP, PMSF, E-64, EMI, pepstatin A, p-chloromercuriphenyl sulfonate, and iodoacetic acid were from Sigma Chemical Co. (St. Louis, MO).³ N,N’-Methylenbis-acrylamide, agarose, and the protein assay kit were from Bio-Rad (Richmond, CA), and all other reagents were of reagent grade or higher.

Grain Germination (Malting)

Barley (Hordeum vulgare L. cv Morex) grain, in 170-g aliquots, was submerged in 16°C water for 36 h (the steeping process of malt production). During steeping, the grain was removed from the water twice, for 1 h each time. At the end of steeping, the grain contained 45% moisture. The steeped grain was immediately placed in a germination chamber and germinated in the dark, with slow rotation, at 16°C and 100% humidity. Aliquots were removed after 1.0, 2.0, 3.0, 4.0, and 5.0 d of germination and were kept frozen at −20°C until analyzed. The 4- and 5-d-germinated material was called green (unkilned) malt. It must be noted that the material had undergone 36 h of steeping before the germination procedure was started. Material designated “1-d-germinated” had, therefore, started imbibing water 60 h previously (36 h steeping and 24 h germinating).

Preparation of Germinating Barley Extracts

Typically, 100 g of material was homogenized in 0.05 M sodium acetate buffer, pH 4.7, containing 2 mM cysteine and 1 mM EDTA and centrifuged, and the supernatant was dialyzed against 5 mM sodium acetate buffer at pH 5.0 as described previously (29). This “crude extract” was frozen in 0.5-mL portions and stored at −20°C.

Roots, shoots, scutellum, endosperm, and aleurone fractions were carefully dissected from 5-d-germinated barley caryopses (4.6 g). Each tissue was then ground with sand in 10 mL of the pH 4.7 acetate buffer cited above. The ground samples were held at 4°C for 1 h and then centrifuged for 30 min at 10,000g. The samples were dialyzed against 20% PEG solution in 5 mM sodium acetate buffer, pH 5.0, which simultaneously concentrated and dialyzed them. The amounts loaded per electrophoresis well were: roots and shoots (analyzed together), 10 μg of protein; endosperm, 18 μg; aleurone, 11 μg; scutellum, 16 μg. Protein concentrations were determined using the Bio-Rad kit.

Endoproteinase Activity Assay in Solution

The proteinase activity of the crude extract was measured in solution, in 2 mM cysteine, using hemoglobin, gelatin, and a hordein extract as substrates. A modification (29) of the method used by Phillips and Wallace (21) to measure hordein hydrolysis was used for all substrates. After reaction, the non-TCA-precipitated peptide amino groups were determined by the ninhydrin reaction described by Lee and Takahashi (16). The enzymic activity was calculated as moles of leucine released per 0.1 mL of extract per hour.

Hydrolases of hemoglobin and hordein were at pH 3.8 in 0.05 M sodium acetate; gelatin hydrolyses were at pH 6.5 in 0.05 M sodium phosphate. For inhibitor studies, blanks were run together with each sample by adding TCA to the reaction mixture before the enzyme.

Hordein Substrate Preparation

Hordein proteins were extracted by our modification (29) of the method of Phillips and Wallace (21). A 55% isopropanol extract was prepared in the presence of 1% β-ME. The final supernatant was incorporated directly into the electrophoretic gels.

For use as a substrate for assaying endoproteolytic activities in solution, the 55% isopropanol-soluble material was precipitated with water and freeze dried. This powder was dispersed in water and homogenized with a Brinkmann Polytron to a homogenous suspension that was added to the hydrolysis mixture.

Electrophoretic Activity Assays

Electrophoresis

Electrophoretic separations were performed using our modification (29) of the method of Heussen and Dowdle (8), the main difference being that SDS was omitted. Samples were applied to the gel in Tris-HCl buffer, pH 6.8. Electrophoresis was run at 4°C and a current of 10 mA per (8.5 × 10 × 0.15 cm) gel for 4.5 h. Either 0.1% gelatin (8), edestin (17), or 0.04% hordein was incorporated into the gels.

³ Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.
Detection of Endoproteinases after Electrophoresis

After electrophoresis, the gels were incubated at 40°C in 0.05 M sodium acetate buffer, pH 3.8, for 3 h or in 0.05 M sodium phosphate buffer, pH 6.5, for 18 h. Both incubation buffers contained 2 mM cysteine. After incubation, the gels were stained with amido black, destained with 30:10:60 (v/v/v) methanol:acetic acid:water, and stored in the destaining solution.

Electrophoretic Data Collection and Processing

Stained gels were scanned with a soft laser densitometer (Biomed Instruments Inc., model SLR-1D/2D) using 620-nm laser light, and the data were analyzed with software from the same company as described earlier (29). The background was electronically subtracted from all scans.

Inhibitor and Activator Studies

For determining the influence of class-specific endoproteinase inhibitors on enzyme fractions, the enzymes were incubated with inhibitors at 0°C for 0.5 h. Stock inhibitor solutions of EDTA, EMI, p-chloromercuribenzoate, iodoacetic acid, and E-64 were prepared in water, DIC and 1,10-phenanthroline were prepared in DMSO, DFP was prepared in isopropanol, and pepstatin A was dissolved in ethanol. Final inhibitor concentrations used in the assays were: DFP, 1 mM; PMSF, 5 mM; DIC, 0.1 mM; E-64, 10 μM; pepstatin A, 20 μM; EDTA, 10 mM; 1,10-phenanthroline, 1 mM; iodoacetic acid, 10 mM; and EMI, 10 mM. The samples were then assayed for total proteolytic activity using the test tube assay or with the electrophoretic assay. For the electrophoretic assay, inhibitors were added to barley extracts either before electrophoresis only (30-min incubation, 0°C) or both before and after electrophoresis. When inhibitor solutions were prepared in DMSO, isopropanol, or ethanol, separate control gels were run that contained the organic solvent but not the inhibitor. To study the effect of reducing agents, gels were incubated, after electrophoresis, either without reducing reagents or with 5 mM β-ME, 2 mM cysteine, or 8 mM DTT.

RESULTS

Temporal Pattern of Appearance of Endoproteolytic Enzymes

Electrophoresis of crude extracts prepared from ungerminated barley grain and from that germinated for various periods from 36 h (steeped) to 5 d beyond steeping revealed that, with gelatin as substrate (Fig. 1A), proteolytic activity was essentially absent until the second day of germination. From the second to the fifth germination days, the proteolytic activity increased until, after 5 d of germination, six proteolytic bands were obvious. These results were obtained when the postelectrophoresis incubation was in 0.05 M sodium acetate, pH 3.8. The endoproteolytic activity bands were numbered from B1 to B6 (Fig. 1, A and C) as described earlier (29). Enzymes were identified on the basis of the ratio of the distance each band migrated to the distance B6 migrated. To ensure that none of the bands detected were due to microbial contamination, two experiments were conducted. In the first, husks (palea and lemma) were removed from 5-d-germinated material and analyzed for proteinase activity. The husk extract showed no proteinase activity, although it should have contained a large proportion of any microbial contamination. In a second test, the germinated grain was treated with 20% bleach to kill and/or wash away contaminating microbes. Analysis of the treated grain did not reveal any loss of proteinase bands. The bands observed thus appear to be from the barley and not from contaminating microbes.

When gels were analyzed that contained immobilized hordein that was initially solubilized in 55% isopropanol under reducing conditions (Fig. 1D), essentially the same pattern was found except that five activity bands were identified with material germinated for 5 d. Because of the low activity levels found with hordein, the gels had to be incubated overnight. When edestin was used, however, proteolytic activity was detected at pH 3.8 in ungerminated and steeped barley (Fig. 1B).

To quantify the proteolytic activities detected in the gels, they were scanned using the normalization methods described previously (29). Because previous studies by Every (7) had shown that densitometric peak areas were not linearly proportional to enzyme concentrations because of diffusion effects, peak heights were analyzed. The band B3 enzymic activity, as determined by densitometric peak height analysis, declined by 47% during the steeping process and the first day of germination, then increased 2.6-fold during germination days 2 to 4, and remained stable through the 5th day of germination (Fig. 1B).

Figure 1. PAGE of barley extracts prepared from grain at different germination stages. U, Ungerminated; S, steeped; 1, 2, 3, 4, 5, days of germination. Thirty microliters was loaded per well. Gels contained incorporated substrate proteins: A and C, 0.1% gelatin; B, 0.1% edestin; D, 0.04% hordein. Incubation occurred in 5 mM sodium acetate buffer, pH 3.8, for either 3 h (A and B) or overnight (D) or in 50 mM sodium phosphate buffer, pH 6.5, overnight (C). All incubation buffers contained 2 mM cysteine. Y axis, Enzyme band designations.
During germination, other activity bands varied as follows (Fig. 1):

1. *Ungerminated Barley.* Very small amounts of the enzyme(s) making up bands B1 and B2 were observed (gelatin, pH 6.5, Fig. 1C).

2. *Germination Day 1.* B1 activity increased strongly (gelatin, pH 6.5, Fig. 1C).

3. *Germination Day 2.* B2 activity increased. B1 activity reached maximum at pH 6.5 (Fig. 1C). At pH 3.8, two B1 enzymes are obvious (Fig. 1A). B3 and B5 became visible (gelatin, pH 3.8, Fig. 1A). With hordein substrate, the activities of all of the enzymes were very low, only traces of B3 and B5 being apparent (Fig. 1D).

4. *Germination Day 3.* B6 was detectable so that all acidic enzymes were present on the gel (Fig. 1, A–C). Most other activities increased.

5. *Germination Day 4.* No new proteolytic bands appeared, although all bands except B1 were stronger (Fig. 1C). One of the two activities previously present in B1 disappeared (gelatin, pH 3.8, Fig. 1A). B6 was the most active enzyme present (gelatin, pH 3.8, Fig. 1A).

6. *Germination Day 5.* The activity of all enzymes was nearly the same as on day 4, except that there was a surge in B4 activity (gelatin and edestin, pH 3.8, Fig. 1, A and B). This band appeared to consist of two separate activities (Fig. 1B).

Data from proteolytic enzyme activity assays conducted in solution (not in electrophoretic gels) using hemoglobin, hordein, and gelatin as substrates were in agreement with the findings obtained with electrophoretically separated endoproteinas, although only the total activities of the crude extracts were measured (Fig. 2, A–C, controls). Hemoglobinolytic activity at pH 3.8 (Fig. 2A, solid line) began to increase on the second germination day, increased 5-fold by day 4, and then remained stable through day 5. Hordein-hydrolyzing activity (Fig. 2B) increased from the 2nd germination day on, but the maximum activity obtained was still only 30% of that found with hemoglobin. With gelatin substrate at pH 6.5 (Fig. 2C), activity increases were not seen until the 3rd day of germination. The total activity with gelatin, pH 6.5, was only 34% of that with hemoglobin at pH 3.8 when 5-d activities were compared. This agrees with our previous data obtained from 4-d-germinated material (29). The activity assay with soluble gelatin was apparently less sensitive than that using gelatin-incorporated electrophoretic gels because the activities of B1 and B2 on gels were easily detected after germination days 1 and 2, respectively, but no increase in total activity was detected in solution until germination day 3.

**Inhibitor and Activator Studies**

**Inhibition of Total Extracts**

The influence of class-specific inhibitors on the total proteolytic activity of extracts prepared from grain at different germination stages was studied first to determine which inhibitors should be tested with the electrophoretic assay. Hemoglobin and hordein (pH 3.8, acetate buffer) and gelatin (pH 6.5, phosphate) were used as substrates in the presence of 2 mM cysteine. E-64 and the thiol-modifying reagents iodoacetic acid and EMI were used to inhibit cysteine proteinases. All three cysteine proteinase-inhibiting compounds strongly inhibited those enzymes responsible for the sharp activity increase seen after the 2nd day of germination (Fig. 2, A–C). They did not block the low levels of activity seen in ungerminated, steeped, and 1-d-germinated grain.

Concentrating on the results obtained with extracts from 5-d germinated material, we found the following: (a) At the concentrations tested, the most potent inhibitor was iodoac-
hemoglobin
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trol
was more
activity decrease


gelatinolytic
or
moglobin-endoproteinases,
gelatinolytic
PMSF
with

(b)
activator
respectively)
(30

acid,

(A,
(B,
10

EMI
and DIC

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Inhibition of Electrophoretically Separated Endoproteinases

Acidic Endoproteinases. Inhibition of electrophoretically separated enzymes was also analyzed. Two cysteine proteinase inhibitors, EMI and E-64, were tested. Enzyme extracts were incubated with the inhibitor on ice for 30 min. After electrophoresis, the gels were incubated with inhibitor at the same concentrations used to study inhibition in solution at pH 3.8. E-64 caused almost complete inhibition of the enzymes digesting gelatin, B1 and B2 showing only faintly (Fig. 3A). B2 activity in the presence of E-64 was essentially the same as in the control (Figs. 1A and 3A). With edestin-containing gel, only one enzyme band (B3) showed much activity in the presence of E-64 (Fig. 3B). The activity of the band, estimated by densitometry, was half that of the control (comparing 5-d germinated samples). This indicates the possibility that B3 contains two or more edestinase activities, not all of which are inhibited by E-64. All of the other edestinases were completely inhibited, except that there was some very low activity in the B1 and B2 regions. Similar results were obtained with EMI (Fig. 3, C and D), although inhibition of some faster migrating enzymes was not complete at the inhibitor concentration tested (20 mM). Bands B3 to B6 were inhibited by between 58 and 84%, whereas neither B1 nor B2 activity was impaired.

The effects of reducing agents, which normally protect cysteine proteinases, were studied. After electrophoresis, the gels were incubated at pH 3.8 in buffer solution (control), 2 mM cysteine, 5 mM β-ME, or 8 mM DTT (Fig. 3E, each analysis being duplicated). Densitometric analyses indicated that only B6 was appreciably stimulated by addition of cysteine or β-ME. DTT, however, increased the activities of all of the enzymes except B1. It is interesting that B2 activity increased 2-fold in the presence of DTT (Fig. 3E), even though it was not inhibited by either E-64 or EMI.

Pepstatin inhibition was studied with gels containing gelatin (Fig. 4A) and edestin (Fig. 4B) incubated at pH 3.8 in the presence of 2 mM cysteine and 20 μM pepstatin. The extracts were also preincubated with 20 μM inhibitor (30 min, on ice) before electrophoresis. The gelatin and edestin gels showed very similar results. There was no gelatinase or edestinase activity in the caryopses until the 2nd germination day, indicating that pepstatin inactivated the edestinase activity before electrophoresis.

Figure 3. Influence of cysteine proteinase inhibitors and activators on electrophoretically separated endoproteinases. Inhibitors were E-64 (A and B, 10 μM) and EMI (C and D, 20 mM); activators (E) were cysteine (2 mM), β-ME (5 mM), and DTT (8 mM). Substrates were gelatin (A, C, and E) or edestin (B and D). Extracts were as listed for Figure 1. Before electrophoresis, samples were incubated with inhibitor (30 min, 0 °C). After electrophoresis, gels were incubated with inhibitor in 0.05 M sodium acetate buffer, pH 3.8, containing 2 mM cysteine (3 h, 40 °C). For activation studies (E), reducing agents were added only after electrophoresis. Con, Control for activator studies, no reducing agent.

cetic acid, inhibiting 74% of the activity of 5-d-germinated grain. (b) EMI and E-64 inhibited hemoglobin hydrolysis by 45 and 44%, respectively (Fig. 2A). (c) The acidic enzyme activities against hordein were even more sensitive to inhibition; iodoacetic acid, E-64, and EMI caused 84, 71, and 62% inhibition, respectively (Fig. 2B). (d) pH 6.5 gelatinolytic activities were also blocked by iodoacetic acid and E-64 (52 and 51%, respectively) (Fig. 2C).

DFP and DIC were used to inhibit serine endoproteinases and caused a relatively low level inhibition (about 20%) with hemoglobin substrate at pH 3.8 (data not shown). The hordein-hydrolyzing activity decrease at pH 3.8 was even smaller, whereas inhibition of the pH 6.5 gelatinolytic activity was more pronounced, 30% inhibition with DFP and 74% with PMSF (data not shown). EDTA, an inhibitor of metal ion-activated endoproteinases, had no influence on the hemoglobin- or hordein-hydrolyzing activities at pH 3.8 or on gelatinolytic activity at pH 6.5. A second inhibitor of metalloendoproteinases, 1,10-phenanthroline, had no effect on the enzymes active at pH 3.8 but caused a 50% inhibition of gelatinolytic activity at pH 6.5 (data not shown).

Figure 4. Inhibition of electrophoretically separated endoproteinases by 20 μM pepstatin. Extracts were as listed for Figure 1. Substrate proteins were gelatin (A) and edestin (B). Samples were treated with pepstatin following the procedures listed in Figure 4 for inhibitors.
proteinases were further lyzed found previously in ungerminated barley. The other endoproteinases were apparently not affected by the inhibitor.

Because of its interesting behavior, enzyme B3 was analyzed further. Densitometric scans of B3 were made on control gels and on gels incubated with either E-64 or pepstatin (Fig. 5). The gels contained edestin and were incubated at pH 3.8. The total B3 activity decreased during steeping and germination day 1 and then increased through day 5. The B3 activity not inhibited by E-64 (the upper part of the band) decreased during the first 3 d of germination and then increased, until on germination day 5 it returned to the level found in ungerminated barley. On germination day 3, a new enzyme, not inhibited by pepstatin, appeared and its activity increased through day 5.

At 2.5 mM, the serine proteinase inhibitor PMSF had no effect on either edestinase or gelatinase activities at pH 3.8. Neither 1,10-phenanthroline nor EDTA affected the acidic proteinases, and there was no increase in enzymic activity when either Mg²⁺ or Ca²⁺ was added to the incubation medium containing 0.05 M acetate buffer, pH 3.8, and 2 mM cysteine.

Neutral Endoproteinases. The influence of class-specific inhibitors on electrophoretically separated neutral proteinases was studied at pH 6.5. Of the inhibitors tested, only DFP showed inhibition (Fig. 6), and it inhibited B1 activity by 40% compared to control samples containing isopropanol. PMSF caused only insignificant (8%) inhibition. DFP caused only a 12% decrease in the activity of B2, and PMSF had no effect (Fig. 6). Metalloproteinase inhibitors influenced neither B1 nor B2 activities. The phosphate salts of many divergent cations are water insoluble; therefore, for testing the influence of Ca²⁺, Mg²⁺, and Zn²⁺ on the enzymes, the phosphate buffer was replaced by citrate buffer, pH 6.5. Metal ions (10 mM) did not alter the enzyme activities. The only effect was that in the presence of each of the three cations the heavy B2 split into three poorly resolved bands. To test the possibility that B1 and B2 might each contain multiple enzymes and that this was masked by overloading the gel, serial dilutions of 4-d-germinated grain extract were electrophoresed in the absence of metal ions and analyzed. The bands became progressively fainter with dilution, but there was no indication of separation into subfractions.

Localization of Endoproteinases in 5-d-Germinated Barley Caryopses

As shown in Figure 7A, aleurone, scutellum, and endosperm tissues, but not shoots and roots, contained acid cysteine proteinases. However, the tissues differed significantly in their enzyme complements. The B6 enzymes were mainly contained in the aleurone tissue, whereas the B5 enzymes were the most prevalent ones in the endosperm. The scutellum contained representatives of all of the enzyme bands except the enzymes at the very front of B6. Shoots and roots did not contain any cysteine proteinase. The pH 3.8 B2
activity of lane 4 in Figure 7A is not due to a cysteine protease(s), as was shown in Figure 3A. Of the neutral proteases, the aleurone layer and endosperm contained almost exclusively band B1 enzymes. The scutellum, shoots, and roots contained both B1 and B2 bands with the B2 activity prevailing, especially in the shoots and roots (Fig. 7B).

**DISCUSSION**

It has been suggested that the process of storage protein mobilization in germinating barley grain involves three phases (6). In the first, proteins contained in the protein bodies of the scutellum and aleurone tissues are hydrolyzed. The second phase involves hydrolysis of the main starchy endosperm storage proteins, and the third involves the breakdown of small peptides resulting from the second phase into amino acids.

The first phase has not been studied in detail in barley (6). Recently, the presence of two aspartic proteases in ungerminated barley grain was reported by Siuro et al. (27) and Kervinen et al. (25). One had a pH optimum between 3.5 and 3.9 and did not digest the unreduced forms of either hordeothionin or β-tryptophan (J. Kervinen, personal communication). Results of this study confirm the presence of aspartic protease(s) in ungerminated barley. We extracted it without having to add either high concentrations of salt or detergents to the extractant. The previously cited authors were not able to detect any aspartic protease except in extractions using high salt concentrations and detergents. The aspartic protease (inhibited only by pepstatin A, of the inhibitors tested) did not hydrolyze gelatin or hordein but effectively digested edestin (in the electrophoretic assay) and hemoglobin (in test tube assays). The edestin hydrolysis decreased by 80% as the pH was increased from 3.8 to 4.7.

We found little or no endoproteinase activity that could correspond to that postulated by Rastogi and Oaks (23), who observed hordein degradation with ungerminated barley half seeds, even in the absence of GA3 stimulation.

There was a second protease(s) (B1) that was active during the early stages of germination. In contrast to the aspartic protease, which was present in ungerminated barley and whose activity declined during steeping and the first 2 d of germination, the B1 activity increased sharply on germination day 1. B1 activity was detected at pH 6.5. The enzyme(s) of this band did not hydrolyze edestin but readily digested gelatin (29). B1 was not significantly inhibited by any of the inhibitors tested, which may indicate that it contains multiple enzymes. In dicotyledonous plants, metalloproteases are responsible for the early stages of protein hydrolysis (26), but similar enzymes have not yet been reported in cereal grain. The role(s) and characteristics(s) of the B1 protease(s) we detected remains to be elucidated. It will be necessary to separate them further before individual enzymes can be studied and characterized.

The second germination phase begins with a sharp increase in the total proteolytic activity. This research showed that the total proteolytic activity of germinating barley, as measured with soluble substrate, increased significantly from the 2nd day of germination when measured at pH 3.8 with hemoglobin and from the 3rd day with hordein substrate (Fig. 2). At pH 6.5 with gelatin substrate, the activity increase began on the 3rd germination day. Similar activity patterns were found by Bhatti (1) with hemoglobin at pH 3.8 and α-N-benzoyl-DL-arginine-p-nitroanilide at pH 8.6 and by Mikola (18) using gelatin at pH 5.4. Our study revealed four pH 3.8 proteolytic activity bands whose activities increased from the 2nd day of germination. Although some of the activities (B6 on gelatin and B4 and B6 on edestin) consisted of two activity bands, they are not discussed separately. It is not yet known whether they are isoenzymes or totally different enzymes. These bands contain proteases of the cysteine protease class, because they were inhibited totally by E-64 and partially by EMI. Both of these inhibitors and iodoacetic acid prevented the germination day 2 endoproteinase activity increase normally found with test tube assays at pH 3.8. This activity surge is induced by GA3 (13).

Two barley cysteine proteases have been purified and characterized, one with an Ms of 30,000 (22) and a second with an Ms of 37,000 (11). The former was synthesized by excised aleurone layers 3 h after GA3 stimulation and the latter was synthesized 9 h later (13). Western immunoblot analysis of the Ms 30,000 endoproteinase showed that it first appeared on the 2nd day of germination (10). From this and earlier (29) studies, it appears that the Ms 30,000 endoproteinase comigrates with B6. Using the electrophoretic separation system, we first detected B6 on germination day 3, which is probably a reflection of the lower sensitivity of this method. It was not possible to determine which of the electrophoretic bands contained the Ms 37,000 endoproteinase because our methods differed from those of the original researchers (11, 13). When nondenaturing electrophoresis was used with gels containing hemoglobin, this protease was detected only on the 4th day after GA3 stimulation (11). In this study, all of the cysteine endoproteinases appeared simultaneously, on the 2nd or 3rd day of germination, except for B4, which was detected only on the 5th day.

All bands identified as cysteine proteases by the use of inhibitors were stimulated by DTT, but only B6 activity was increased by cysteine or β-ME when applied at concentrations normally causing optimal stimulation (9, 21). This may reflect the fact that the oxidation-reduction potential of DTT is higher than that of either β-ME or cysteine (14).

Cysteine proteases have previously been implicated in the splitting of insoluble endosperm hordeins into soluble peptides (6). The major natural substrates for these enzymes are the barley storage proteins, among which the hordeins predominate. Hordeins are prolamins and are thus insoluble in water but are extractable with organic solvents such as 70% ethanol or 55% isopropanol, especially under reducing conditions. Their insolubilities in aqueous buffers make them convenient substrates for incorporation into electrophoretic gels. When we conducted analyses in a pH 3.8 solution with a hordein suspension, very little proteolytic activity was detected in an ungerminated grain extract. Hordein suspension hydrolysis with extract from 5-d-germinated barley was 10-fold higher than that of ungerminated grain extract. This is similar to the results obtained by Phillips and Wallace (21). Extracts of 4- or 5-d-germinated barley yielded, on electrophoresis, five proteolytic activity bands that hydrolyzed reduced hordein. No hydrolysis of hordein isolated under
nonreducing conditions was detected, even with extracts from 4-d-germinated material (29).

β-Hordeins, the main (80–90%) barley prolamin components, have relatively high cysteine contents, which presumably contribute to intramolecular and intermolecular disulfide bonds (24). It may be that reduction of hordein sulfhydryl groups determines their susceptibilities to endopeptidases and that the reducing characteristics of the cellular environment may strongly affect endopeptidase activity. This possibility is supported by the recent findings of Buchanan (2) with the barley seed thioredoxin. His findings suggested that thioredoxin may play a regulatory role during germination by reducing disulfide bonds of both storage proteins and selected enzymes. On germination day 5, the cysteine proteinases were located mainly in the aleurone and endosperm tissues and were absent in the shoot and root tissues. This lends support to the idea that cysteine proteinases are responsible for at least some of the mobilization of storage proteins during germination.

In addition to cysteine proteinases, we also detected aspartic proteinase(s) activity that increased during germination. When the development of this proteinase (B3) during germination was monitored in edestin-containing gels incubated with cysteine proteinase activity blockers, the aspartic endopeptidase activity decreased until the 3rd germination day and then increased, reaching its original (ungerminated grain) value on germination day 5.

In addition to the four activity bands containing acidic endopeptidases, we also detected two enzymes whose activities increased with increasing pH. At pH 5.5 and 6.5, no cysteine or aspartic endopeptidase activity was detected; only bands B1 and B2 were visible. These two activity bands were barely detectable at pH 3.8. Both B1 and B2 activities were active after SDS was removed with Triton X-100, as long as the samples had not been reduced or boiled (29). These high mol wt enzymes readily digested gelatin, but not edestin, in the electrophoretic assay. Band B1 activity was inhibited by DFP and PMSF. This is consistent with the fact that the total endopeptidase activity (measured in solution) with gelatin (pH 6.5) was inhibited by both serine and cysteine proteinase inhibitors. The less than total inhibition of B1 and B2 probably reflects their both containing multiple proteolytic enzymes.

B1 activity increased beginning from the 1st germination day and reached its maximum on day 2. B2 appeared simultaneously with other endopeptidases on the 2nd germination day. Enzymes with characteristics similar to B1 and B2 have been described previously. Enari and Mikola (5) found that there were some barley endopeptidases that had basic pH optima but were inhibited by EDTA. The B1 and B2 proteinases were insensitive to cation-chelating agents. Burger (3) detected two proteinases that he called neutral proteinases. These have characteristics similar to B1 and B2 and may be the same, but this needs to be confirmed.

The roles of the neutral proteinases contained in B1 and B2 are not known. The B2 enzyme(s) were found exclusively in plantlet parts that developed from the embryo. It has been postulated that small peptides formed by cysteine proteinases in the endosperm are taken into scutellum and are digested there into free amino acids by neutral aminopeptidases (6). If the neutral proteinase(s) that we detected also take part in this process or whether they are only involved only in embryo metabolism remains to be elucidated.

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LITERATURE CITED

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